Attorney's Docket No. 5218-39A PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: **Application of Anagnostou and Sigounas**

Serial No. **08/842,700** Group No. 1642

Filed: **15 April 1997** Examiner: S. Ungar

For: **Method of Treating Endothelial Injury** Date: 2 November 1998

RULE 132 DECLARATION OF DR. GEORGE SIGOUNAS

The Honorable Commissioner of
Patents and Trademarks
Washington, DC 20231

Sir:

I, George Sigounas, Ph.D., do hereby declare and say as follows:

1. I received my Ph.D. from Boston University in Cellular Biology. I am currently Professor of Medicine at East Carolina University School of Medicine in Greenville, North Carolina. I am a co-inventor on the above-identified patent application.

2. The following experiments were carried out under my direction. These experiments were designed to assess the ability of erythropoietin (EPO) delivered concomitantly with a platinum coordination complex chemotherapeutic agent, to inhibit endothelial cell proliferation in tumors, and thus inhibit tumor vascularization and suppress tumor growth. Cisplatin was used as the platinum coordination complex chemotherapeutic agent in these studies; cisplatin is a known antineoplastic chemotherapeutic agent (available as PLATINOL®, Bristol-Myers).

3. Lewis lung carcinoma cells were injected subcutaneously in mice to produce tumors. The animals were divided into five groups of five mice per group; the groups were treated as indicated in **Table 1**. Epo and cisplatin were injected intraperitoneally. The concentrations used were 60 U per mouse (EPO) and 10 mg/kg/mouse (cisplatin). Tumor volume was measured daily using Vernier Calipers. Twelve days after Lewis lung carcinoma cell inoculation, all animals were sacrificed by cervical dislocation. The tumors were excised, weighed, observed macroscopically, fixed in 4% formaldehyde, and embedded in paraffin. Histological sections (5μm thickness) were stained with Mason's trichrome and examined under a microscope for blood vessel formation. Tumor neovascularization was evaluated from the stained sections at 10X magnification. 4X and (65, 119198)

Table 1

Treatment Protocol

Day	Group 1	Group 2	Group 3	Group 4	Group 5
0	-	-	-	-	-
1	-	EPO	-	EPO	-
2	-	-	-	-	-
3	-	-	Cis	Cis	Cis/EPO
4	-	EPO	-	EPO	-
5	-	-	-	-	-
6	-	-	Cis	Cis	Cis/EPO
7	-	EPO	-	Epo	-
8	-	-	-	-	-
9	-	-	Cis	Cis	Cis/EPO
10	-	-	-	-	-
11	-	-	-	-	-
12	-	-	-	-	-

Cis = cisplatin 10 mg/kg each cycle; intraperitoneal injection
EPO = erythropoietin, 60U/mouse each cycle; intraperitoneal injection
Mice = 5/group
Animals in Group I were injected with PBS alone.

4. The weight of excised tumors was compared (see **Table 2**, where a 1-fold reduction indicates no difference in weight; a 2-fold reduction indicates a 50% reduction in weight, etc.). As shown in **Tables 2** and **3**, animals in Group 1 (PBS alone) or Group 2 (EPO alone) had the largest tumors. The average tumor mass in animals injected with cisplatin alone (Group 3) was reduced 3.8-fold compared to Group 2 (EPO alone).

5. In Group 5 animals (simultaneous EPO/cis), tumor mass was reduced 4.5-fold compared to Group 2; this reduction is greater than that seen in Group 3 (cisplatin alone). See **Table 2**.

6. Animals injected first with EPO and two days later with cisplatin (Group 4) showed the greatest reduction in tumor mass. Tumor mass was reduced 8-fold compared to the tumors observed in Group 2. See **Table 2**.

Table 2

Change in Tumor Mass

Group	Treatment	Final Tumor Weight Reduction*
2	Epo x 3	1.0-fold
3	Cisplatin x 3	3.8-fold
4	EPO/Cisplatin x 3 (sequential)	8.0-fold
5	EPO/Cisplatin x 3 (concomitant)	4.5-fold

* compared to PBS injected animals (Group 1)

7. Additionally, a reduction in tumor volume was also observed in animals that were (a) treated simultaneously with Epo and cisplatin (Group 5) or (b) treated first with Epo and then with cisplatin (Group 4); compared to tumor volume in Group 2 or Group 3. See **Table 3**.

Table 3

Change in Tumor Volume

Group	Treatment	Tumor Volume (mm^3)					
		d-5	d-6*	d-7**	d-8	d-9*	d-10
2	Epo x 3	108	149	217	256	368	569
3	Cisplatin x 3	78	126	134	175	248	171
4	Epo/Cisplatin x 3 (sequential)	81	122	160	126	122	98
5	Epo/Cisplatin x 3 (concomitant)	31	45	76	106	166	156

* cisplatin injection for Groups 3 and 4; Epo/Cis injection for Group 5.

**Epo injection for Groups 2 and 4.

8. Histological analysis of tumor sections demonstrated that tumors excised from animals treated with Epo alone were highly vascularized and contained small and large vessels (pink to red color in Figure 1). See **Figures 1** and **4**. Tumor sections from animals injected with cisplatin alone contained fewer vessels and capillary-like structures (**Figures 2** and **4**) compared to tumors excised from Epo treated animals (Group 2) (**Figures 1** and **4**). Tumor sections from animals treated with Epo and Cisplatin (Group 4) showed greatly reduced vascularity; portions of the tumors became necrotic and remaining tumor cells were only loosely connected (**Figures 3** and **4**).

9. The studies described above indicate to me that erythropoietin (EPO), added before or concomitantly with a chemotherapeutic agent, can inhibit endothelial cell proliferation in tumors and enhance the chemotherapeutic agent's ability to suppress the growth of tumors.

10. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

George Sigounas

George Sigounas, Ph.D.

11/9/98

Date

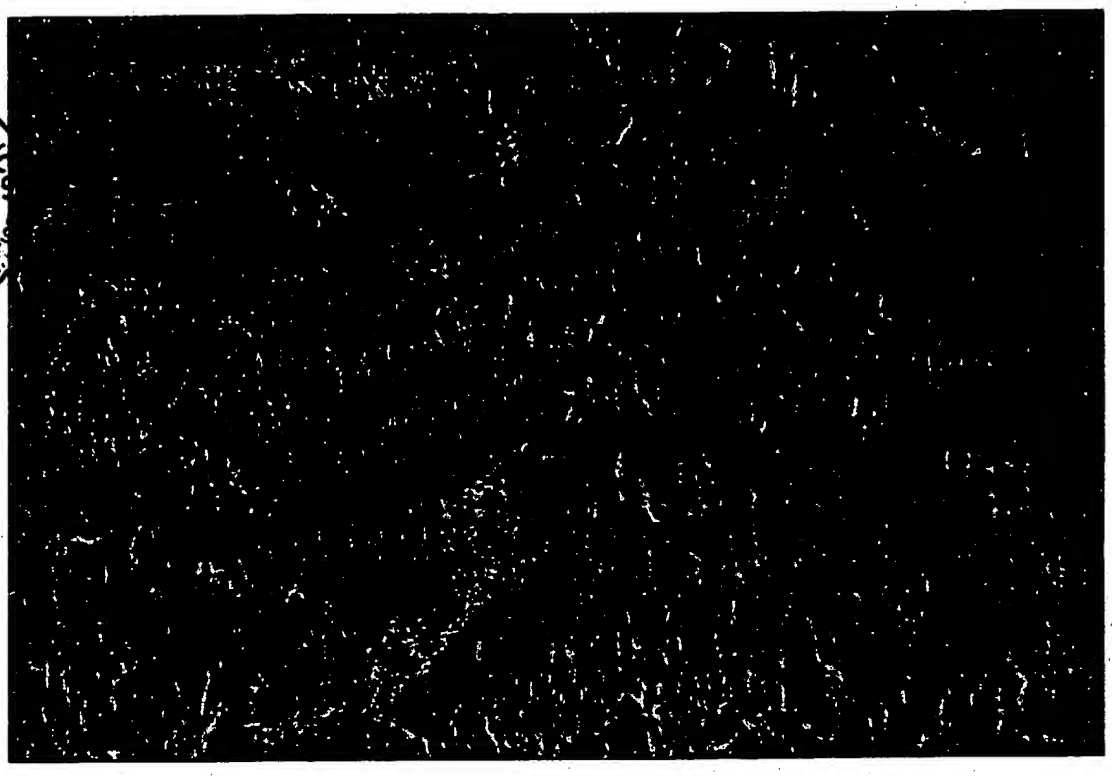

Fig. 1

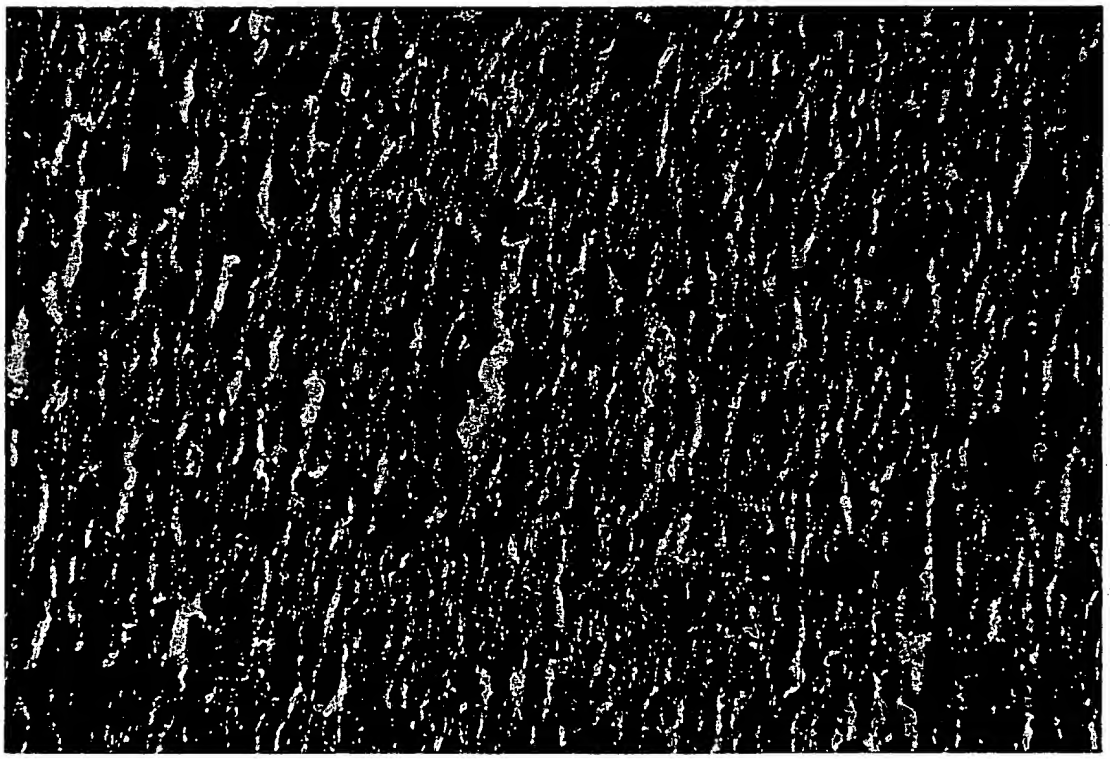

Fig. 2

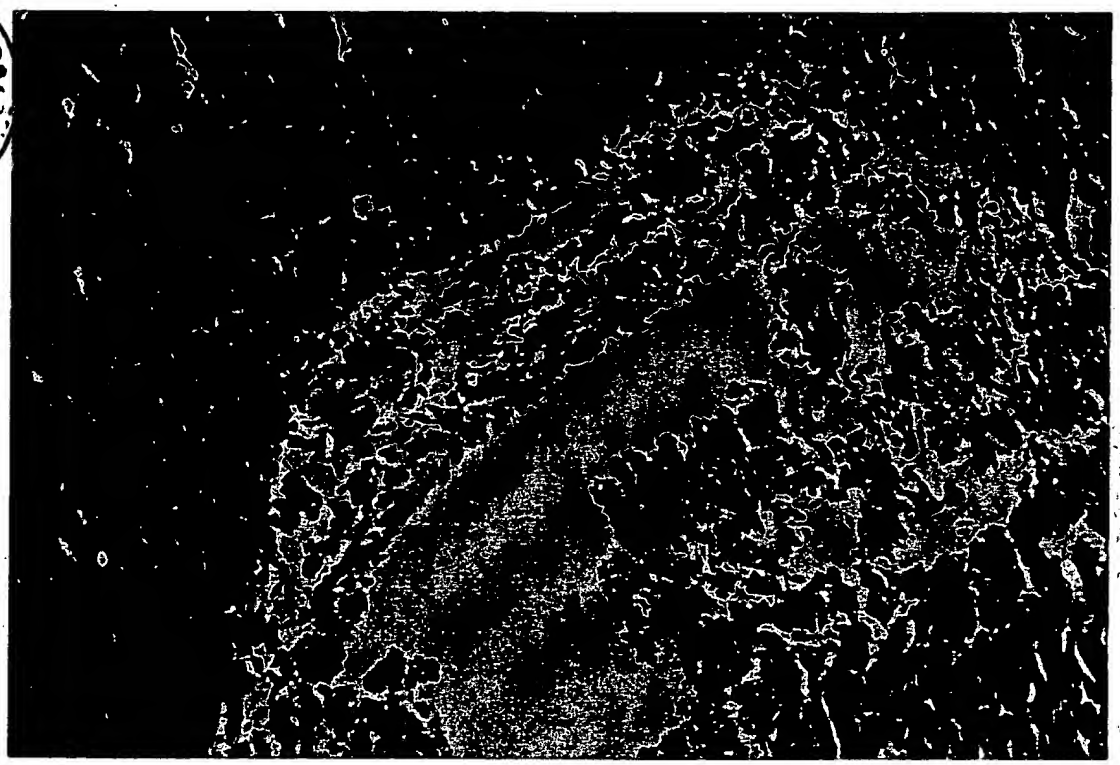

Fig. 3

Effect of Epo and/or Cisplatin on Tumor Vasculature

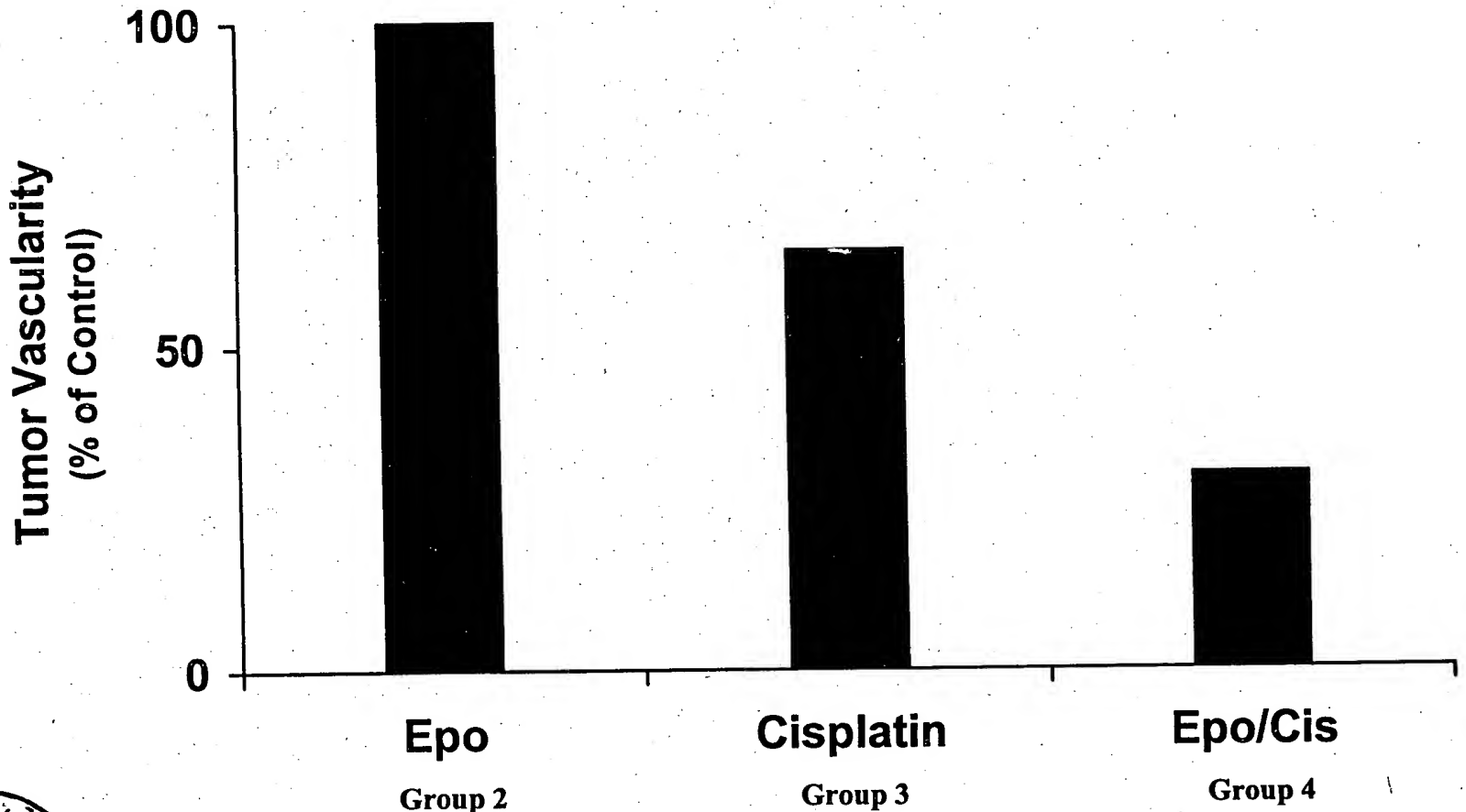

Figure 4